



EFFECT OF SALINITY AND DROUGHT STRESS ON GROWTH PARAMETERS, GLYCOSIDE CONTENT AND EXPRESSION LEVEL OF VITAL GENES IN STEVIOL GLYCOSIDES BIOSYNTHESIS PATHWAY OF *Stevia rebaudiana* (BERTONI)

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Abstract- *Stevia* [*Stevia rebaudiana* (Bertoni)], a family of Asteraceae is known to yield diterpenoid steviol glycosides (SGs) that is about 300 times sweeter than sugar. The present work examined the effects of different concentrations (25, 50, 75 and 100mM) of NaCl and mannitol on growth, physiological response, glycosides content and expression profile of four genes in steviol glycosides biosynthesis pathway after 30 days of culture. The results showed that NaCl and mannitol significantly ($p < 0.05$) affect the no. of shoot, shoot length, root no. and root length. As NaCl and mannitol concentration increases leaf DW, stem DW, root DW, shoot DW and leaf FW were decreased markedly by 30-70 % and 50-55 %, respectively at 100mM. In addition, transcript expression profiling of genes involved in steviol glycoside biosynthesis pathway showed an increased in expression genes; KAH, UGT85C2, UGT74G1 and UGT76G1 in 50, 75 and 100mM NaCl concentrations compared with control. Interestingly, expression ratio of UGT76G1 (Rebaudioside A) was significantly increased by 67% as compared to UGT74G1 (Stevioside), showing that salinity stress plants are sweeter than normal plant, whereas all four genes were down-regulated in drought stress conditions. These results were compared with the steviol glycoside contents measured in the leaves that was quantified by HPLC. Results showed that amount of Rebaudioside A was significantly increased to 45.83, 63.32 and 80.21 mg g⁻¹ at 50, 75 and 100mM NaCl condition whereas significant decrease was found in all treatment condition of mannitol. Present work thus suggests that NaCl is acting as an enhancer and mannitol acting as a repressor of transcription to genes of steviol glycoside biosynthesis pathway that could alter the production of steviol glycosides.

Keywords- Salinity stress, drought stress, *S. rebaudiana*, Steviol glycosides, Real-time quantitative PCR, HPLC

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Introduction

The spiraling popularity of plant-derived healthcare products can be traced to the advent of green health boom across the world. India also known as "home of Ayurveda" has been a potential market for *Stevia Rebaudiana*. As a medicinal plant it is poised to open up new exciting avenues in healthcare.

Stevia Rebaudiana Bertoni, belongs to the family of Asteraceae. It is a perennial and endemic, medicinal shrub [1]. *Stevia* is a natural non-calorie sweetener [2] and recommended for diabetes as well as extensively tested on animals. It is also used by humans with no side effects [3]. *Stevia* is the ideal sweetener for those who suffer from hypoglycemia, high blood pressure, diabetes, obesity and chronic yeast infections.

The leaves of *Stevia* contain glucosides i.e. stevioside and rebaudioside with a sweet taste. The biggest part of the sweet glycosides consists of the stevioside molecule [4]. The sweetener, stevioside [5] extracted from plants is 300 times sweeter than sugar. About nine active components of steviol glycosides (SG) which are they,

stevioside (SV), rebaudioside A (RbA), rebaudioside B (RbB), rebaudioside D (RbD), rebaudioside F (RbF), steviolbioside (Stb), rubusoside (Rub), rebaudioside C (RbC) and dulcoside A (DuA). Each of them contributes their own percentage of sweet flavor to the *Stevia* leaf.

Micropropagation can provide genetically uniform plants in huge numbers. However, with climate change, a huge quantity of salt and alkali land have been occurred that possessed excellent light conditions for the growth and secondary metabolites accumulation of *S. rebaudiana*. Moreover, water deficit and salt stress are global issues to ensure survival and sustainability of industrial important crops. Previous research reports alterations in biomass, antioxidant enzyme activities and osmolytes, mineral contents and secondary metabolites while plants under salt stress [6-7]. Moreover, development of new variety of *S. rebaudiana* with a higher content of rebaudioside-A and a reduced content of stevioside is the primary and main aim of plant breeders concerned with the improvement and utilization of this source of natural sweeteners. Several experiments

were designed to measure the transcript levels of downstream genes contributing to the biosynthesis of steviol glycosides. Using qRT-PCR, expression level of three UDP-dependent glycosyltransferases, UGT85C2, UGT74G1 and UGT76G1, were studied at different time interval of *Stevia Rebaudiana*. Three candidate genes, UGT74G1, UGT76G1 and UGT85C2, of which the translation products were found to have an activity towards steviol glycosides, were cloned [8]. The addition of the C-13-glucose to steviol is catalyzed by UGT85C2, the C-19-glucose by UGT74G1 and finally glucosylation of the C-3 of the glucose at the C-13 position is catalyzed by UGT76G1. UGT74G1, UGT76G1 and UGT85C2 belong to family GT1 of the glycosyltransferase group according to the CAZy classification (<http://www.cazy.org/fam/GT1.html>). The plant CAZy family 1 substrates include terpenoids, alkaloids, cyanogenic glucosides and glucosinolates as well as different form of flavonoids [9].

The present study proposes to consider the effect of NaCl and mannitol on growth, physiological response, glycosides content and expression profile of four genes involved in steviol glycosides biosynthesis pathway in relation to their accumulation patterns after 30 days of *in vitro* regenerated *S. rebaudiana* (Bertoni).

Materials and Methods

Plant Material and Experimental Conditions

In vitro regenerated plant of *Stevia Rebaudiana* (Bertoni) was maintained in plant tissue culture lab of Xcelris Genomics, Ahmedabad at $27 \pm 2^\circ\text{C}$ under 2,000-2,500 lux for 16 h/day. *In vitro* salinity and drought stress was performed on proliferating shoot after 30 days of subculture. For this, cluster of multiple shoots were separated and re-inoculated on MS medium supplemented with 1 mg/l BAP + 0.5mg/l Kn [10] and different concentration of NaCl (0, 25, 50, 75 and 100 mM) and mannitol (0, 25, 50, 75 and 100 mM) for salinity and drought stress, respectively. These growing explants were harvested after 30 days of culture. Furthermore, 2 gm of leaves were harvested in which 1 gm was dried for HPLC analysis and 1 gm was frozen immediately in liquid nitrogen and stored at -80°C until further use for gene expression analysis.

Morphological Response

The plants exposed to varying concentration of NaCl and mannitol was analyzed after 30 days for evaluation of plant development. The morphogenetic responses of the explants were recorded up to 15 passages. Each experiment was repeated three times with 12 replicates for each treatment. The results were recorded in the form of Mean \pm Standard Error (SE). The growth parameters was calculated as mean number of shoots (MNS), mean shoot length (MSL), mean number of root (MNR) and mean root length (MRL). The data recorded were analyzed statistically using two-way ANOVA

(analysis of variance) and comparisons between the mean values of treatment were made by using Duncan's multiple-range test ($p < 0.05$).

Gene Expression Analysis

Gene expression was studied to evaluate the influence of variable concentration of NaCl and mannitol on steviol glycoside biosynthesis pathway. For this, 3 genes encoding UDP-glycosyltransferases (UGT76G1, UGT85C2 and UGT74G1) and 1 gene KAH play a significant role in steviol glycoside biosynthesis pathway, was selected. Total RNA was isolated from leaf tissue after 30 days old culture having different treatment concentration of NaCl and mannitol as well as control plant using Qiagen RNeasy Plant Mini Kit as per manufacturer's instructions. The quantification and qualification of total RNA was done using Qubit fluorometer and 1 % denaturing agarose gel electrophoresis, respectively. Further, cDNA was synthesized from 1 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) as per manufacturer's instructions. 2 μl of synthesized cDNA was utilized as template for qRT-PCR using LightCycler 480 II instrument (Roche).

Five gene specific primers were designed from accession ID i.e. AY345974, AY345978, AY345982, EU722415 and KJ813746 using FastPCR software [Table-1]. 18s RNA was used as a control. The optimized PCR conditions were: 95°C , 5 mins for 1 cycle; 95°C , 10 sec; 58°C , 15 sec; 72°C , 20 sec for 35 cycles. Three replicates were run for all five genes with each sample. Primer efficiency of genes and their relative expression was calculated using relative expression software tool (REST $^{\text{C}}$) as described by Pfaffl et al [11].

Quantification of Steviol Glycosides

For analysis of steviol glycosides (SVglys), 100 mg of freeze-dried leaves of both salinity and drought stress conditions with different concentrations were extracted using methanol: water (80:20, v/v) at room temperature for 12h. The obtained extracts were filtered and vacuum dried. Dried extracts were defatted with hexane and the resultant residual matter was vacuum dried. These extracts were further dissolved in acetonitrile:water (80:20, v/v), filter sterilized and processed for HPLC analysis. 10 μl was injected in HPLC using LiChrospher $^{\text{R}}$ NH2 phase column (25 cm \times 3.2 mm, 5 μm particle size, UV detection at 205 nm) using an isocratic solvent system of acetonitrile:water (80:20, v/v). The column temperature was maintained at 25°C and the flow-rate was 0.8 ml min^{-1} [12]. The stevioside and rebaudioside A were identified by comparison of their retention times with standards. To prepared calibration curve, solutions containing 0.1-0.5 mg m^{-1} of each standard in acetonitrile:water (80:20, v/v) were used. Each analysis was repeated three times and mean value was used.

Table 1- Primer sequences and their calculated efficiency of target and housekeeping genes using REST $^{\text{C}}$ software

Gene Name	Sequence 5'-3'	Accession ID	PE \pm SE'
UDP Glycosyltransferase (UGT76G1)	F primer: CCCGGTACCATTCAAGGCCACA R primer: GGAATCCGCATACCAGCGAGC	AY345974	2.1 \pm 0.0
UDP Glycosyltransferase (UGT85C2)	F primer: ACACCGACTTCATCCACAACCA R primer: GCAGCGTCAATTGTGAAAACCGA	AY345978	2.1 \pm 0.0
UDP Glycosyltransferase (UGT74G1)	F primer: GGTGAAACATGGACCCGAACAAG R primer: GGCATTGCAACAACGGGGACTCC	AY345982	2.0 \pm 0.0
Kaurenoic acid-13 hydroxylase (KAH)	F primer: AGAGATTTGTTTCGTGAACGGATCAAG R primer: TCCATGGTGACGGCATAATGAGTTGC	EU722415	2.0 \pm 0.1
18S	F primer: GTCTCAACCATAAACGATGCCGACCA R primer: ACCTGGTAAGTTTCCCGTGTGAGT	KJ813746	2.1 \pm 0.0

'PE \pm SE': Primer efficiency \pm Standard error

Results and Discussion

Fundamentally, plants require energy (light), water, carbon and mineral nutrients for growth. Abiotic stress is defined as environmental conditions that reduce growth and yield to the normal levels. Response of plant to abiotic stresses is dynamic and complex [13-14]; they are both elastic (reversible) and plastic (irreversible).

Effect of NaCl and Mannitol on Plant Morphology

Plant growth inhibition is a phenomenon often occurring in glycophyte or salt-sensitive plant species under salinity and drought stress conditions. Earlier, *in vitro* regeneration protocol was developed for *Stevia Rebaudiana* 'Bertoni' [10]. In the present investigation, *in vitro* regenerated plantlet was used for salinity and mannitol stress study. Results showed that salt and drought stress induced significant differences on plant growth after 30 days of experimental period. The significant decrease in the shoot number and shoot length was observed with the increasing concentration of NaCl (25, 50, 75 and 100mM). The highest number of shoot (~17) and shoot length (9.9 cm) was observed at 25mM after 30 days and lowest number of shoot (~9) and shoot length (7.4 cm) at 100mM after 30 days of treatment as compared with control [Fig-1], [Fig-2]. The plants grown under control (no salinity application) exhibited the maximum values for shoot number (~20) and shoot length (11.1 cm) as compared to remaining treatments after 30 days of culture, which indicated that salinity is responsible for reduction in shoot number and shoot length [Table-2]. Shahid et al [15] observed the reduction in shoot length with increasing concentration of NaCl.

This might be explained as the inadequate photosynthesis caused by stomatal closure and the reduction of carbon assimilation rate under salt stress [6]. According to the view of Maas and Hoffman [16] it was suggested that *S. rebaudiana* is a moderately salt-tolerant plant.



Fig. 1- *S. rebaudiana* grown under normal conditions (control)



Fig. 2- Effect of salinity stress on plant growth and development at different concentrations 25 (a), 50 (b), 75 (c) and 100mM (d) of NaCl after 30 days of culture

Drought is a multidimensional stress affecting plants at various levels of their organization [17-18]. The response to drought at the whole plant level is complex because it reflects the integration of stress effects and responses at all underlying levels of organization over space and time [19]. In the present investigation, drought stress exhibited significant differences on shoot number and shoot

length during the experimental conditions. After 30 stress days, significant decreases in shoot number and shoot length was observed in plants grown with 25, 50 75 and 100mM mannitol. The means values of shoot number and shoot length were 15.7, 13.7, 9.3, 8.0, 4.3 and 10.5, 9.5, 8.8, 7.6, 7.7 cm for treatments of 0, 25, 50, 75, and 100mM mannitol, respectively [Table-3], [Fig-3].



Fig. 3- Effect of drought stress on plant growth and development at different concentrations 25 (a), 50 (b), 75 (c) and 100mM (d) of mannitol

Table 2- Effect of different NaCl concentration on growth parameter of *in vitro* generated *S. rebaudiana* after 30 days of treatment^a

NaCl (mM)	MNS	MSL	MNR	MRL	Leaf Number	Shoot/root ratio	g plant ⁻¹				
							Leaf DW	Stem DW	Root DW	Shoot DW	Leaf FW
0	20.0±1.15a	11.1±0.16a	10.3±0.33a	7.4±0.7a	55.3±0.33a	11.1±0.28a	0.87±0.01a	0.37±0a	0.18±0.003a	1.27±0.001a	5±0.1a
25	17.7±0.33ab	9.9±0.11ab	7.7±0.33ab	6.1±0.1b	44.7±0.33ab	9.8±0.23ab	0.85±0a	0.31±0ab	0.14±0.003 b	1.05±0.06ab	4±0.06ab
50	15.3±0.33bc	9.1±0.19bc	6.3±0.33bc	5.2±0.03bc	35.3±0.33bc	7.7±0.2bc	0.73±0.01b	0.27±0.01 bc	0.12±0.003bc	0.79±0.02 bc	3.3±0.08bc
75	12.0±0.57cd	8.1±0.1cd	4.7±0.33cd	4.8±0.02bc	24±0.58c	6.2±0.2c	0.69±0.01bc	0.23±0 cd	0.09±0.003 c	0.58±0.03 c	2.7±0.11cd
100	9.3±0.67d	7.4±0.07d	3.3±0.33d	4.1±0.06c	22.3±0.33c	5.1±0.17c	0.64±0c	0.19±0 d	0.08±0.006 c	0.48±0.02 c	1.6±0.01d

^aData are the mean±SE (n = 3). Different letters following values in the same column indicate significant difference among salt treatments using Duncan's multiple-range test at p < 0.05.

Salinity and drought stress also exhibited significant differences on root number and root length. *In vitro* regenerated stress induced shoots were transferred to rooting medium containing ½ strength MS medium supplemented with 2 mg/l IBA. With the increasing concentration of NaCl and mannitol significant decrease in root number and root length were observed after 30 days. Interestingly, at 50 and 75mM NaCl and 75 and 100mM mannitol significant decrease in root length was not observed [Table-2], [Table-3]. Similar

result was observed by Abdel-Hussein [20] in which MM106 and Omara apple rootstocks for salt tolerance *in vitro* was evaluated. This reduction might be due to the inhibitory effects of salt on the metabolic activities which associated with cell division, differentiation and elongation [21]. Ashraf et al. [22] reported that plants grown under control (no salinity application) exhibited the maximum values (10.11 cm) for root length as compared to remaining treatments that indicate that salinity is responsible for reduction in root

length. Thiam et al [23] reported that root length was adversely affected with a significant reduction of NaCl concentration (50 and 100 mM) and reached 6.95 cm and 6.33 cm, respectively. Their findings revealed that high NaCl concentration significantly affect the root growth. Nayar & Gupta [24] also reported that drought stress significantly reduces the root length in various plant species wheat and maize.

Moreover, salinity and drought stress significantly decreased leaf fresh weight (FW), leaf dry weight (DW), stem DW, shoot DW and

root DW after 30 days of treatment [Table-2], [Table-3]. Zeng et al [25] reported that plants treated with 90 and 120 mM NaCl showed significantly lower plant height, leaf number, branch length, stem dry weight, leaf fresh/dry weight, and shoot dry weight than control. Srivastav & Srivastav [26] also presented the similar findings that severe drought stress conditions reduce the plant biomass of *S. rebaudiana*. These results were similar to the findings of Pirzad et al [27] and Mohammadian et al [28].

Table 3- Effect of different mannitol concentration on growth parameter of *in vitro* generated *S. rebaudiana* after 30 days of treatment^b

Mannitol (mM)	MNS	MSL	MNR	MRL	Leaf Number	Shoot/root ratio	g plant ⁻¹				
							Leaf DW	Stem DW	Root DW	Shoot DW	Leaf FW
0	15.7±0.33a	10.5±0.18a	10.7±0.33a	6±0.12a	47±0.58a	9.4±0.09a	0.82±0.01a	0.3±0.01a	0.17±0.01a	1.3±0.01a	5±0.1a
25	13.7±0.33ab	9.5±0.17ab	9.3±0.33ab	5.2±0.15ab	43.3±0.33ab	7.2±0.09b	0.77±0.01a	0.26±0ab	0.16±0a	1.3±0.01a	4.7±0.03a
50	9.3±0.33bc	8.8±0.08bc	6.7±0.33bc	4.3±0.06bc	37±0.58bc	5.4±0.12bc	0.56±0.01b	0.25±0.01b	0.14±0ab	1.2±0a	4.2±0.09ab
75	8±0.58cd	7.6±0.02cd	5±0cd	3.7±0.07c	31.3±0.33cd	4.8±0.06c	0.53±0.01b	0.23±0.01b	0.13±0.01b	0.8±0.01b	3.4±0.12bc
100	4.3±0.33d	7.1±0.08d	3.3±0.33d	3.4±0.06c	25.7±0.67d	4.2±0.09c	0.45±0.01b	0.17±0.01c	0.09±0.01c	0.7±0.01b	2.7±0.12c

^bData are the mean±SE (n = 3). Different letters following values in the same column indicate significant difference among salt treatments using Duncan's multi-range test at p < 0.05.

Gene Expression Analysis

The leaves of *Stevia Rebaudiana* (Bertoni) accumulate numerous steviol glycosides (SVglys), the concentrations of which can vary widely depending on the genotype, growth conditions and developmental stage [4, 29]. Changes in the content of carbohydrates such as glycosides, together with the unbalanced of plant hormones function as a signal that plant response to stress should be initiated [8], possibly involved in the protection against ROS that are responsible for loss of membrane integrity and cellular death. Moreover, induction of sugar accumulation, i. e., glycosides is relevant for the osmoprotection process and has been associated to plant tolerance to water stress [30]. In otherwise, plant defense response and tolerance to drought and salinity involves the perception of signal stress by receptors at the membrane level followed by signaling transduction in the cell, inducing a multiplicity of biochemical mechanisms involved in the protective role of secondary metabolites. There are number of genetic approaches available to investigate the action of the glycosyltransferases in the plant and how their catalytic activities may be related to physiological functions.

SGs are derived from MEP pathway involving series of enzymes encoded by the corresponding genes. Three UGTs (UGT85C2, UGT74G1 and UGT76G1) are known to modulate the synthesis of stevioside and rebaudioside A and hence assumes importance [8]. In order to have an account of NaCl and mannitol variability on steviol glycoside biosynthesis pathway, the transcript expression of four genes of the pathway was analyzed; KAH (Kaurenoic acid 13-hydroxylase) and three UGTs (UDP-glycosyltransferases) UGT85C2, UGT74G1 and UGT76G1. Relative concentration of each gene was calculated with p-value. Results showed that expression of KAH was significantly increased at each concentration (50, 75 and 100mM) of NaCl excepting 25 mM where KAH expression was repressed but expression ratio was not significant (P < 0.05). On the other hand, exposure of plant to 50, 75 and 100mM NaCl significantly (P < 0.05) up-regulated the expression of UGT85C2, UGT74G1 and UGT76G1 while 25mM NaCl concentration significantly (P < 0.05) repressed all three UGTs. More interestingly, when we compared the expression ratio of UGT74G1 and

UGT76G1 with control, significant increase of rebaudioside A (r = 3.13) was observed that was nearly to stevioside (r = 2.46) at 100mM concentration [Fig-4]. The alteration in transcript expression profile in response to varying NaCl suggested the NaCl responsiveness of steviol glycoside biosynthesis pathway related genes.

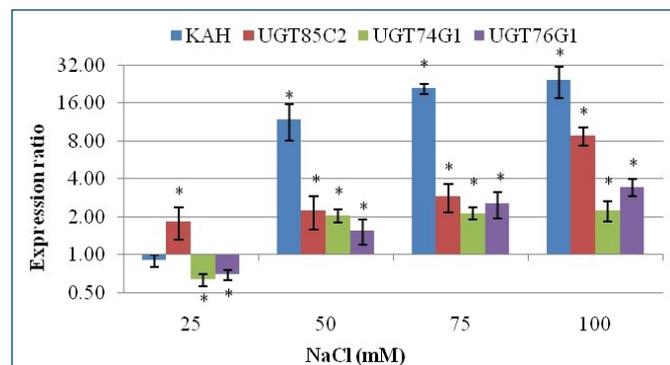


Fig. 4- The expression of the three UGTs and KAH relative to control in leaves at 25, 50, 75 and 100mM NaCl treatments. All the experiments were repeated three times. Each value represents the mean±standard error of three separate biological replicates *Significant expression (p < 0.05). Normalization to 18S rRNA is not shown.

At molecular level, expression ratio of UGT85C2, UGT74G1 and UGT76G1 might be induced by varying concentration of NaCl. Over expression of some glycosyltransferase genes led to a significant increase in their respective glucosides [31-32]. In general, it is difficult to correlate a specific UGT and a specific derivative where plant UGTs are thought to be not highly specific and show broad substrate specificity based on a regio-selectivity [8, 33-34]. However, the significant correlation between UGT85C2 transcription and total SVgly accumulation suggesting that the UGT85C2 enzyme, which adds a C-13-glucose to steviol to form steviolmonoside, is the rate-limiting step of the glycosylation pathway during SVgly biosynthesis. The reason why this correlation was only found at 50, 75, and 100 mM NaCl concentration might be because gene transcription and SVgly accumulation is a result of plants reaction with increasing

concentration of NaCl that enhances their ROS scavenging capacity compared with control. Also, this transition might be stressful for the plants [35] possibly resulting in a higher production of more reactive oxygen species (ROS). Stoyanova et al [36] reported that SVglys are very potent ROS scavengers.

In case of drought stress conditions, all four genes were significantly ($P < 0.05$) down-regulated at 25 mM mannitol concentration. Moreover, significant regulation was not found at 50 mM of mannitol in UGTs group except KAH that were significantly down-regulated. At 75mM mannitol, UGT76G1 was significantly down-regulated. UGT76G1 is majorly involved in RebA production in the pathway. However, regulation of UGT85C2 and UGT76G1 were significantly down-regulated at 100mM mannitol [Fig-5]. Eventually, results showed that all four genes were significantly down-regulated at each treatment concentrations of mannitol treated plant.

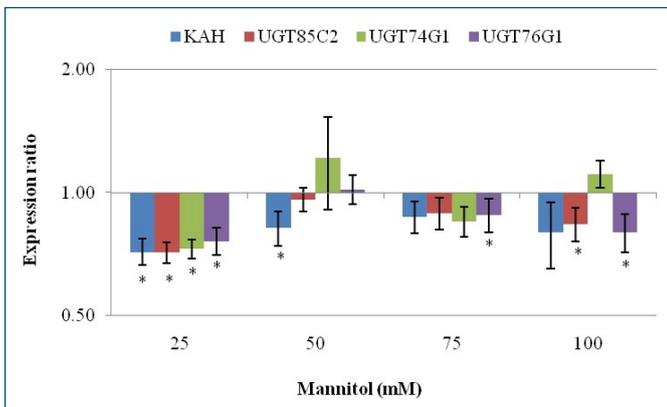


Fig. 5- The expression of the three UGTs and KAH relative to control in leaves at 25, 50, 75 and 100mM mannitol treatments. All the experiments were repeated three times. Each value represents the mean±standard error of three separate biological replicates *Significant expression ($p < 0.05$). Normalization to 18S rRNA is not shown.

Earlier research suggests that transcription patterns of SVglys biosynthetic genes varied with different treatments. Similar results were also observed by Hajjhashemi et al [37] in which transcription

of UGT85C2 and UGT76G1 significantly decreased in PEG treatments. They also reported that transcription of KAH and UGT74G1 was not affected under PEG treatments while opposite results were observed in our studies whereas both genes were significantly down-regulated under drought stress conditions. Earlier research by Hajjhashemi et al [38] reported that stevioside content decreased in PEG treatments, whereas the transcription of UGT74G1 was not significantly affected by treatments. However, Reb A content and UGT76G1 transcription both noticeably decreased under PEG treatments. Our results suggest that change in Reb A content is due to its precursor and UGT76G1 transcription; increased Reb A content in salinity tolerant plant while decrease Reb A content in drought tolerant plant resulting negatively effect on genes transcription and SVglys accumulation under drought stress.

Quantification of Steviol Glycosides

Stevia plants have gained importance as sweeteners because of their stevioside (ST) and rebaudioside (Reb) contents. Earlier it was believed that ST is the main sweet component, but recent research reports that Reb is responsible for the sweetening properties of stevia leaf. In context of HPLC analysis, determined the level of stevioside and conversion of ST to Reb A under salinity and drought stress plant. In the experimental sample significant change in the level of ST and Reb A was observed with respect to control indicating the functional nature of the enzyme under stress conditions. Different concentrations of NaCl and mannitol were used for determining the glycoside content but only NaCl stress condition showed positive results. Data from HPLC analysis of ST, Reb A and ratio of ST and Reb A are shown in [Table-4] for salinity stress and [Table-5] for drought stress as mg g^{-1} . Results showed that under different concentrations of NaCl, the accumulation of ST were significantly ($p < 0.05$) higher compared with control plant except 25mM NaCl where ST accumulation was significantly decreased. In general, Reb A accumulates in more or less constant ratio to ST (about two times more ST than Reb A) but Reb A was significantly increased 45.83, 63.32 and 80.21 mg g^{-1} at 50, 75 and 100 mM NaCl concentrations, respectively in this study.

Table 4- Effect of different concentrations of NaCl on leaf glycoside contents of *S. rebaudiana*¹

Glycosides (mg g^{-1})	NaCl				
	Control	25 mM	50 mM	75 mM	100 mM
ST	36.69±1.22cd	32.47±1.14d	41.03±0.9bc	45.05±0.93ab	52.46±0.53a
Reb A	28.59±0.28c	22.94±0.27c	45.83±0.15bc	63.32±0.09ab	80.21±0.29a
Reb/ST	0.78±0.23cd	0.71±0.23d	1.12±0.16bc	1.41±0.1ab	1.53±0.55a
ST + Reb A	65.29±1.5c	55.41±1.41c	86.86±1.05bc	108.36±1.02ab	132.66±0.82a

¹Data are the mean±SE (n = 3). Different letters following values in the same row indicate significant difference among NaCl treatments using Duncan's multiple-range test at $p < 0.05$.

Table 5- Effect of different concentrations of Mannitol on leaf glycoside contents of *S. rebaudiana*¹

Glycosides (mg g^{-1})	Mannitol				
	Control	25 mM	50 mM	75 mM	100 mM
ST	36.69±1.22a	28.47±1.14ab	21.65±0.17bc	16.47±0.14cd	10.46±0.13d
Reb A	28.6±0.28a	24.39±0.27ab	17.98±0.41bc	13.8±0.21cd	8.86±0.28d
Reb A/ST	0.78±0.23b	0.86±0.23a	0.83±2.49a	0.84±1.55a	0.85±2.16a
ST + Reb A	65.29±1.5a	52.85±1.41ab	39.63±0.58bc	30.27±0.34cd	19.32±0.4d

¹Data are the mean±SE (n = 3). Different letters following values in the same row indicate significant difference among mannitol treatments using Duncan's multiple-range test at $p < 0.05$.

These results were confirmed with significant increased in ratio of Reb A/ST at 50, 75 and 100mM NaCl concentrations. The results indicated that NaCl could modulate the composition of steviol glycosides for its function of promoting the transformation of stevioside to rebaudioside A. Similar results were also obtained by Zeng et al [25], who found that ratio of RA content, RA/ST, and RA/(RA+ST) increased, whereas ST content and ST/(RA+ST) decreased in plant treated with 60, 90 and 120 mM NaCl treatments. Moreover, our findings also reports that mutant produced by 100mM NaCl stress treatment contain ~2 times more Reb A than ST.

In context of drought stress condition, quantity of ST and Reb A were significantly ($p < 0.05$) decreased at 25, 50, 75 and 100 mM mannitol compared with control. Moreover, ratio of Reb A/ST stats that Reb A content was lower than ST but significant differences was not found as per Duncan's multiple-range test at $p < 0.05$. Similar results were also reported by Hajjhashemi et al [38] in which significant degradation of ST was found in *in vitro* plant treated with polyethylene glycol-induced drought stress. Their study reports that PEG significantly reduces the amount of total SVglys per plant by about 82 %. On the contrary, Behroozi et al [39] reported that drought stress significantly increase the accumulation of SVglys content.

Conclusion

In conclusion, results obtained in the present investigation suggest that exposure of varying NaCl and mannitol concentrations imposes osmotic imbalance around *Stevia Rebaudiana* "Bertoni" plants. The increasing concentrations of NaCl and mannitol during the treatment conditions suggesting that *Stevia Rebaudiana* are least resistant to salinity and drought conditions. The plant underwent various morphological variations on 25 and 100 mM NaCl and mannitol; however, plants grown on 50 and 75 mM NaCl were quiet healthy. Interestingly, the expression profile of genes involved in steviol glycoside biosynthesis pathway was significantly altered during NaCl and mannitol concentrations changes. Increase in NaCl concentrations enhanced the expression of UGTs genes mainly UGT76G1 i.e. involved in Reb A biosynthesis than UGT74G1 while decreases during mannitol treatment conditions. Furthermore, transcript alterations were favoured by the data of quantitative steviol glycoside estimations. Thus, present data suggested that salinity stress markedly enhances the content of Reb A than ST while these contents were decrease in plant grown under drought stress.

Abbreviations

FBB: Frequency of bud break
 FBE: Frequency of bud elongation
 MNS: Mean number of shoot
 MSL: Mean shoot length
 MNR: Mean number of root
 MRL: Mean root length
 FW: Fresh weight
 DW: Dry weight
 PEG: Polyethylene glycol
 KAH: Kaurenoic acid hydroxylase
 SVglys: Steviol glycosides
 ST: Stevioside
 Reb A: Rebaudioside A

UGT: Uridine diphosphate-dependent glycosyltransferase
 HPLC: High-performance liquid chromatography

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